

**PROJECT 1: AN INVESTIGATION OF FcRL4 POSITIVE B
CELLS IN THE RHEUMATOID SYNOVIUM**

**PROJECT 2: THE DEVELOPMENT OF A CULTURED ELISPOT
ASSAY FOR THE DETECTION OF LOW FREQUENCY T CELL
RESPONSES.**

By

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An Investigation of FcRL4 Positive B cells in the Rheumatoid Synovium

Abstract

Rheumatoid arthritis (RA) is a chronic inflammatory disease affecting ~1% of the population. B cells central to disease progression, demonstrated by the potency of anti-CD20 treatment, rituximab. FcRL4 is a transmembrane protein expressed on a subset of memory B cells. FcRL4⁺ B cells which have never been described in RA, characteristically express chemokine receptors CCR1 and CCR5 and cytokine RANKL suggesting a possible role for these cells in chronic inflammatory diseases. Using immunofluorescent microscopy and qPCR we have investigated the prevalence of FcRL4⁺ B cells in the synovial tissue, synovial fluid and peripheral blood of RA patients. FcRL4⁺ B cells were not identified in synovial tissue sections by immunofluorescent microscopy due to weak staining of FcRL4 which could not be detected over the threshold set for matching isotype controls. However, qPCR suggests FcRL4 expression is higher in synovial tissue of established RA patients compared to healthy controls. FcRL4 expression is also elevated in the synovial fluid and peripheral blood of RA patients. Future should focus on optimisation of a suitable staining technique for the detection of these cells and characterisation of their function in RA with a view to development of novel therapies for treatment of this debilitating disease.

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Introduction

Pathogenesis of Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory disease which affects ~1% of the population and is associated with increased co-morbidity and mortality. The disease is characterised by joint pain and swelling, and bone destruction, and is often associated with the presence of autoantibodies[1]. The causes of RA are not fully understood, although genetic factors are thought to contribute ~60% to disease liability [2]. Genetic risk factors such as HLA-DRB1 shared epitope alleles and mutations of tyrosine phosphatase PTPN22 have been identified. These alleles are linked to increased prevalence of anti-cyclic citrullinated peptide antibodies (ACPA) and rheumatoid factor (RF), which are associated with more severe disease and radiological damage[3, 4] [5]. RF is autoantibodies which target the Fc region of IgG immunoglobulins and are commonly used for the diagnosis of RA [6]. PTPN22 is a negative regulator of T and B cell receptor signalling pathways, implicating these cells in disease pathogenesis. While genetic factors are indisputably linked to disease susceptibility, environmental factors are also implicated. Smoking is broadly considered to be a major environmental risk factor for RA. Smoking results in increased T and B cell activation in the lungs and is causally linked to increased ACPA and RF and more severe disease. Trauma or infection can also lead to disease exacerbation through localisation of inflammation to the joint [7].

Elucidating the immune cell populations present in the rheumatoid synovium is vital for understanding the mechanisms underlying disease pathogenesis. Cells of innate and adaptive immune systems interact together and contribute to chronic inflammation of the synovium in RA. Neutrophils are the most abundant immune cell type in humans, but their role in disease progression of RA is often overlooked [8]. Activated neutrophils produce a number of inflammatory cytokine including TNF, IL-1, IL-18, IL-15 and IL-6 which may contribute to the exacerbation of disease. Extracellular traps produced by neutrophils (NETs) are induced by high levels of ACPA

and RF antibodies and enhance inflammation. NETs have also been shown to externalise citrullinated proteins which may further promote ACPA formation [9]. It has also been suggested that neutrophils may play an important role in the deposition of autoantibodies into the joint [10]. Furthermore, *in vivo* mouse models of RA have indicated that neutrophil depletion results in improved prognosis [11, 12].

High numbers of activated macrophages are common in inflamed joints and are thought to be significant effectors of disease via the production of numerous pro-inflammatory and regulatory cytokines and chemoattractants such as IL-1, IL-6, IL-8 and TGF- β [13]. Most significantly, macrophages are key producers of TNF- α which potently increases production of cytokines by synovial cells [14]. Furthermore, macrophage numbers in the synovium of RA patients have been shown to correspond to degree of radiographic damage [15].

Enhanced osteoclast formation is responsible for the articular destruction associated with severe RA. Receptor activator of nuclear factor- κ B ligand (RANKL) is required for osteoclast differentiation to their terminal state, and is expressed by activated synovial fibroblasts and T cells [16, 17]. Cytokines in the rheumatoid synovium such as IL-6 and IL-17 induce RANKL expression [18]. TNF- α , a potent cytokine of RA, is also known to drive RANKL expression and subsequent bone destruction [19]. Monoclonal antibodies to RANKL have been developed and while they reduce the joint destruction associated with RA, inflammation and other symptoms are not reduced.

As previously mentioned, susceptibility to RA is closely associated with mutations of genes linked to T cell function [3, 4] [5]. As such, T cells are traditionally considered to be pivotal in disease progression, although T cell depletion therapy has proven to be largely ineffective [20, 21]. Animal models of RA have suggested inflammatory Th17 T cells to be particularly significant and have

shown regulatory T cells to have impaired function [22-25]. T cells are believed to mediate pathogenicity via direct contact with synovial macrophages and also the secretion of inflammatory cytokines including IL-17 which induces activation of neutrophils, macrophage and local stromal cells thus contributing to an inflammatory loop[22] [26]. In addition, evidence of B cell somatic hypermutation in RA has been found, indicating antigen-specific T cell reactivity in the rheumatoid synovium[26].

B cells in Rheumatoid Arthritis

B cells are often found in inflamed joints and are believed to significantly contribute to the pathogenesis of RA[27]. One important role for B cells in RA is the production of auto-antibodies such as RF and ACPA which are known to exacerbate disease and are associated to a poor prognosis and increased bone destruction [3, 4] [5]. These antibodies may form immune complexes and activate local B cells to produce more auto-antibodies, or secrete cytokines, by binding to Fc and complement receptors on their surface [27].

A crucial role for B cells as a bridge between innate and adaptive immune responses in RA has been described. B cells are able to process and present antigens to T cells in the synovium and induce their activation. This is emphasised by the discovery that depletion of B cells reduced the ability of T cells to produce pro-inflammatory cytokines [28]. T cell activation will result in subsequent activation of synovial macrophages and the exacerbation of disease. In addition, B cells are maintained in the synovium by the production of survival and activation signals such as APRIL (a proliferation-inducing ligand) and BAFF (B-cell activating Factor) by innate cells upon TNF stimulation[22].

B cells are known to produce a plethora of cytokines in response to stimulation, but their cytokine production in RA has not been thoroughly investigated. However, they have been reported to produce pro-inflammatory TNF- α and IL-6. More recently, B cells from synovial fluid of patients with established RA have been shown to produce other potent cytokines including RANKL and IL-12, further highlighting their role in disease pathogenesis [29]. Further studies are required to elucidate the significance of cytokine production by B cells in RA.

Rheumatoid Arthritis – Diagnosis and Treatment

Early diagnosis and treatment of the disease is essential for the prevention of irreversible joint destruction. Current diagnosis is largely dependent on the detection of RF and ACPA antibodies which are only present in ~65% of patients [32]. Therefore many patients go undiagnosed until later stages of the disease when irreversible bone destruction may have occurred[30]. The discovery of prognostic biomarkers broadly expressed in patients with early RA would certainly improve outcomes.

Current treatment involves a combination of anti-inflammatory drugs and targeted immunotherapy. Initially, non-steroidal anti-inflammatory drugs are proscribed, but as disease worsens stronger medications are administered, many with severe side effects. Methotrexate and hydroxychloroquine are steroids which stall disease progression. However, these drugs become ineffective over time and more drastic measures may be required. Biological therapies targeting multiple pathogenic cells and cytokines have been developed with varying success. TNF- α and IL-6 antagonists have been shown to reduce symptoms; however they are expensive and only effective in ~50% of cases. Therefore these treatments are not commonly used[30].

Rituximab is a monoclonal antibody which selectively depletes CD20⁺ B cells (all B cells except plasma cells) and is commonly used for the treatment of RA [31]. Rituximab is effective in ~65% of cases and is particularly useful for the treatment of RF and ACPA positive patients. Interestingly, rituximab treatment has been shown to prevent bone destruction, a finding which further demonstrates the critical role of B cells in disease progression of RA. Manipulation of the immune response with biologics cannot be targeted only to the synovium and therefore patients are often left seriously immunocompromised and susceptible to secondary infection. To avoid this, more targeted treatments must be developed.

FcRL4⁺ B cells

Fc receptor-like 4 (FcRL4) is a transmembrane protein preferentially expressed in a distinct subset of primed memory B cells found in tonsils and other epithelial and mucosal surfaces [32, 33] [34]. FcRL4 is an inhibitory receptor as the intracellular domain is comprised of 3 immunoreceptor tyrosine-based inhibitory motifs (ITIMs) [35]. Indeed FcRL4 prevents BCR signalling by association with tyrosine phosphatases SHP-1 and SHP-2[32]. More recent studies have shown that FcRL4 can acts as an Fc receptor which binds IgA, suggesting a role for these cells in control of immune responses [36].

Despite being classified as memory B cells, FcRL4⁺ B cells are physically larger than FcRL4⁻ memory B cells and lack CD27 expression [37]. Activation of FcRL4⁺ B cells relies on T-dependent activation and cytokine signalling, and results in high levels of antibody secretion [37]. In HIV infected patients, the presence of FcRL4⁺ has been linked to B cell exhaustion [38]. Interestingly, it has been suggested that in response to continued antigen stimulation, this population of B cells may revert to an innate-like cell phenotype, up regulating TLR9 signalling[32].

FcRL4⁺ B cells characteristically express a number of soluble mediators including chemokine receptors CCR1 and CCR5 (suggesting that these cells are likely to localise in chronically inflamed sites) and RANKL (a key cytokine in RA pathogenesis). Additionally, FcRL4⁺ polymorphisms have been implicated in susceptibility to autoimmune ankylosing spondylitis which questions whether this unique population of memory B cells may be implicated in other chronic inflammatory diseases such as RA [39].

Aims

1. To investigate the prevalence and function of FcRL4⁺ B cells in synovial tissue biopsies taken from patients with established and early (prediagnosis) RA, and resolving arthritis using immunofluorescence microscopy and quantitative PCR.
2. To investigate the prevalence of FcRL4⁺ B cells in the synovial fluid and peripheral blood mononuclear cells of patients with established RA using quantitative PCR.

Methods

Clinical samples

In accordance with ethics and following informed written consent, synovial fluid and peripheral blood was obtained from patients in the Birmingham area. Patients suffered from a range of rheumatic diseases including; rheumatoid arthritis, monoarthritis, gout, systemic lupus erythematosus and undiagnosed arthritis. Samples were obtained from a total of 58 patients and 6 healthy donors. Synovial tissue biopsies were taken with ultrasound guidance and immediately frozen in liquid nitrogen. Peripheral blood mononuclear cells (PBMCs) and synovial fluid mononuclear cells (SFMCs) were isolated by density gradient centrifugation prior to this investigation and stored at -80 until use.

Macrophage stimulation

In order to optimise a protocol for TNF- α staining, cytopins were prepared using activated macrophages. PBMCs were isolated from peripheral blood of a healthy donor. CD14 positive monocytes were isolated by MACS by another member of the laboratory. 1×10^6 monocytes were incubated in 1ml RPMI 1640 supplemented with 5% heat inactivated fetal calf serum (HIFCS), penicillin, streptomycin, glutamine and GM-CSF for 6 days at 37 C. LPS (1 μ l/ml) was then added and the cells were incubated for 4 hours at 37 C. The cells were then removed from the plate by scraping. 10,000 cells per slide were applied to the cytopsin. Cells were spun at 3000 RPM for 5 minutes, air dried fixed in acetone at 4 C and stored at -20 until use.

Immunofluorescence microscopy

Immunofluorescence staining was carried out on 5µm synovial tissue biopsy sections from patients with established RA. Tonsil tissue sections were used for the staining optimisation procedure. The sections were cut and stored at -80 C prior to this investigation. In all cases slides were hydrated with PBS for 5 minutes and then blocked for 10 minutes with 10% HIFCS in PBS. The primary antibody was diluted to the appropriate concentration in PBS supplemented with 0.5% BSA, and 100µl was applied to the tissue section (or cytospin). The slides were incubated for 1 hour on a shaking tablet at room temperature. The slides were then washed for 5 minutes in PBS. The secondary antibody was diluted to the correct concentration in PBS supplemented with 0.5% BSA and 100 µl was added to the sections. The slides were then incubated for a further 30 minutes on a shaking tablet at room temperature. Finally the slides were washed for 5 minutes in PBS and mounted using mounting medium which contained nuclear stain. The slides were wrapped in foil and stored at 4 C until use. In an attempt to reduce non-specific binding an Fc blocking reagent was tested. In these cases, slides were blocked with 10% Fc blocking reagent and 10% HIFCS in PBS prior to staining. Negative controls were prepared in the same way using non-specific isotype control antibodies at matching concentrations to the test antibodies. Images were taken using a LSM 510-UV confocal (Zeiss) and a LSM780 Zen microscope (Zeiss).

Mouse anti-CD20 (Dako) and rabbit anti-RANKL (AbCam) antibodies had been previously titrated and optimised and were applied throughout at 3.5µg/ml and 10µg/ml respectively. CD20 was developed with goat anti-mouse FITC (green) (Southern Biotech) and RANKL was developed with donkey anti-rabbit rhodamine (red) (Jackson Immuno Research).

In order to optimise TNF-α staining, a titration was carried out on macrophage cytospins. Cytospins were stained for the macrophage marker CD68 and TNF-α. The mouse anti-CD68 antibody (BD Pharmaceutical Systems) was added at 5µg/ml. The mouse anti-TNF-α antibody (R&D systems)

was tested at 10µg/ml, 5µg/ml and 0.5µg/ml. TNF-α was developed with goat anti mouse TRITC (red) and CD68 was developed with goat anti mouse Cy5 (blue)

Mouse anti-FcRL4 PECy7 conjugated antibody (Biolegend) had been previously optimised and was initially used at 0.25µg/ml. Due to concerns about fluorescence interference, an unlabelled mouse anti-FcRL4 antibody (Biolegend) was also tested. In order to optimise unlabelled FcRL4 staining, a titration was carried out on tonsil sections. The antibody was tested at 10µg/ml, 5µg/ml, 1 µg/ml and 0.5µg/ml. Sections were stained for the B cell marker CD20 and FcRL4. FcRL4 was initially developed with goat anti-mouse Cy5 (blue), but was later developed with goat anti mouse IgG2b Rhodamine (red) so that FcRL4 positive cells could be seen through the eye piece of the confocal microscope.

Anti-CD20, anti-FcRL4, anti-TNF-α, and anti-RANKL antibodies were tested on synovial tissue biopsy sections at 3.5µg/ml, 5µg/ml, 10µg/ml and 10µg/ml respectively.

Real-time quantitative PCR

Q-PCR was carried out in order to determine *FcRL4* gene expression in cells isolated from peripheral blood, synovial fluid and synovial tissue biopsies of patients and healthy donors. RNA was isolated using the Qiagen Mini RNA extraction kit and was subsequently reverse transcribed into DNA using the Superscript Vilo Kit as per the manufacturer's instructions. The reaction was set up in 96 well plates to a total volume of 25µl. 5µl DNA was added to 1µl of the gene assay, 12.5µl Taqman master mix and 6.5µl RNase free water per reaction. All reactions were performed in duplicate. The Applied Biosystems 7900HT PCR machine was used with the following protocol: 2 min at 50C, 10 min at 95C, 45 cycles x [15 sec at 95C, 1 min at 60C]. GAPDH, 18S and beta-actin were tested as potential housekeeping genes using PBMCs isolated from a healthy donor. 18S was selected for subsequent experiments. Primers were obtained from Applied Biosystems.

A titration experiment was carried out to determine the optimal DNA reaction concentration. DNA was diluted according to the PBMC number from which the DNA had been extracted so that DNA from 1×10^6 , 5×10^5 , 2.5×10^5 , 1.25×10^5 or 62.5×10^4 cells were tested per reaction. For investigations of fluid samples (PBMCs and SFMCs) DNA from 1.25×10^5 cells was added per reaction.

For PCR investigations of synovial tissue cell number was unknown and so DNA isolated was applied to the assay undiluted. For analysis of real-time PCR data the threshold was set at 0.2 for all reactions.

Analysis of results

For PCR reactions relative gene expression (RQ) values were calculated using the formula: $RQ = 2^{-(Ct_{FcRL4} - Ct_{18s})}$. To determine distribution differences between test groups, Mann Whitney tests were carried out. $p < 0.05$ was considered to be statistically significant.

Results

A more detailed knowledge and understanding of the immune cells prevalent in the rheumatoid synovium is essential for the development of new diagnostic tools and more targeted treatments for RA. This investigation focuses on the identification of FcRL4⁺ B cells in synovial tissue, SFMCs and PBMCs of healthy donors and patients with RA. Immunofluorescent microscopy enables visualisation of cell localisation within the synovial tissue, while quantitative PCR elucidates the expression of the *FcRL4* gene. Optimisation of the staining and PCR protocols were essential for accurate detection of FcRL4 and reduced non-specific staining. Matching isotype controls for each experiment were set to negative and the settings were reused for imaging the cells of interest. Optimisation stainings were imaged using the LSM 510-UV confocal microscope. The Zen microscope was used for imaging synovial tissue sections as it can be used to quantify staining and co-localisation of cell markers.

Anti- TNF- α antibody should be used at 5 μ g/ml

In order to assess the appropriate concentration of anti-TNF- α antibody to use for staining of synovial tissue biopsies, an antibody titration was carried out on activated macrophages from healthy donors. Macrophage cytopins were prepared and stained for TNF- α (red) and macrophage marker CD68 (blue). Anti-CD68 was added at 5 μ g/ml and anti-TNF- α was tested at 10 μ g/ml, 5 μ g/ml and 0.5 μ g/ml.

Macrophages were strongly intracellularly stained with CD68. TNF was detected in a relatively small proportion of macrophages at all concentrations with intracellular localisation. Staining was relatively weak when anti-TNF- α was applied at 10 μ g/ml compared to 5 μ g/ml (figure 1a,1b). This was due to high background levels seen on the isotype controls (data not shown) at 10 μ g/ml. At

0.5 μ g/ml TNF- α staining was weak (figure 1c). 5 μ g/ml anti-TNF- α was selected for use in subsequent staining investigations.

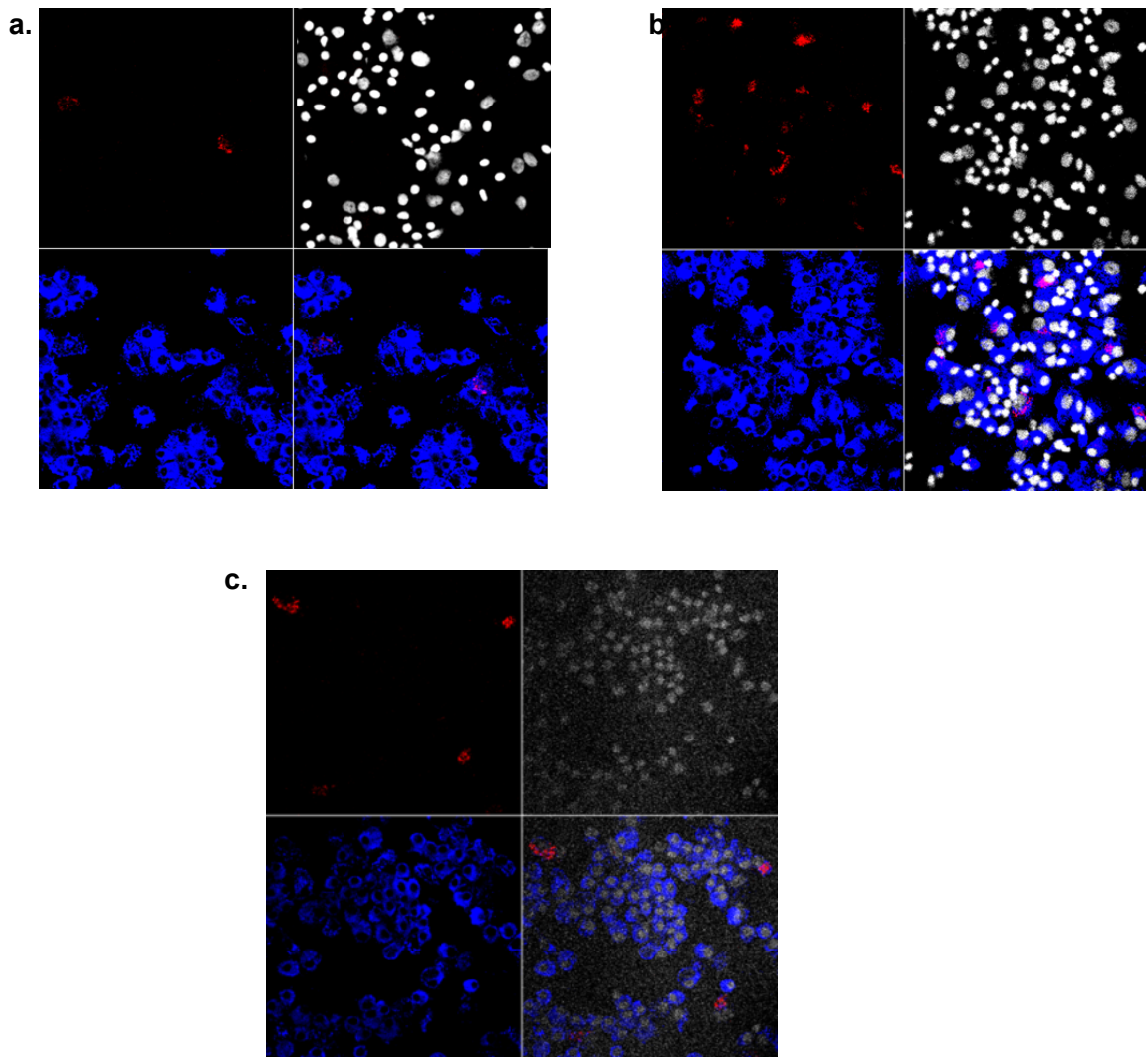


Figure 1. Titration of anti-TNF- α antibody on activated macrophage cytopins. Cytopins were stained for TNF- α (red) and CD68 (blue). Clockwise from the bottom left corner panels show: CD68 staining, TNF- α staining, nuclear counter staining, and the 3 stains overlaid. **(a)** anti TNF- α antibody added at 10 μ g/ml **(b)** anti TNF- α antibody added at 5 μ g/ml **(c)** anti TNF- α antibody added at 0.5 μ g/ml.

Triple positive cells stained for CD20, FcRL4 and TNF- α may be the result of overlapping wavelengths.

CD20 (green), FcRL4 (blue) and TNF- α (red) staining was tested on a synovial tissue sections taken from a patient with established RA, known to have B cell infiltrates in the joint. Anti-CD20, anti-FcRL4 and anti-TNF- α antibodies were added at 3.5 μ g/ml, 0.25 μ g/ml and 10 μ g/ml respectively.

Clear co-staining of FcRL4 and TNF- α was seen on the surface membrane of cells. CD20 staining was present but weak as background was high and so signal was low. Fc blocking reagents should be tested to reduce non-specific antibody binding. FcRL4⁺ cells appeared to be arranged in a wavelike structure which suggests localisation beneath the synovial lining layer, or a blood vessel. All stained cell appeared to be triple positive for FcRL4, CD20 and TNF- α which raises concerns of spectral bleed-through, caused by partial overlap of the excitation or emission spectra of the antibodies used.

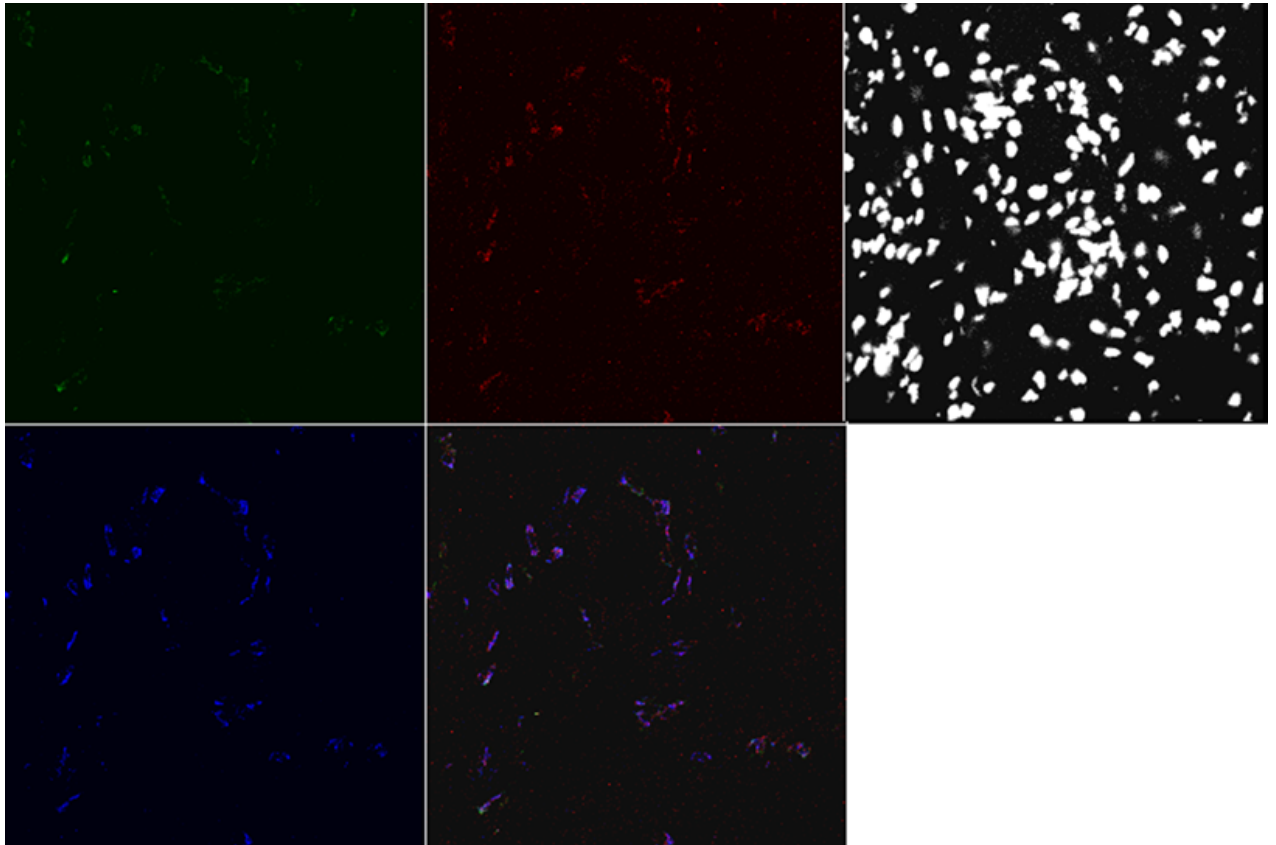


Figure 2. Detection of FcRL4⁺ B cells in synovial tissue. Sections were stained for CD20 (green), FcRL4 (blue) and TNF-α (red). Panels show CD20 staining, TNF-α staining, nuclear counterstain, FcRL4 staining and CD20, TNF-α and FcRL4 overlaid.

Blocking slides with Fc blocking reagent reduces background.

CD20 staining could not be detected on previously stained synovial tissue sections due to high backgrounds. In an attempt to reduce non-specific antibody binding and therefore reduce background levels an Fc blocker was tested. Previous slides were blocked with 10% HIFBS in PBS before staining. During this investigation slides were blocked with 10% Fc blocker and 10% HIFBS in PBS. Slides were then stained with anti-CD20 (green) at 3.5 µg/ml.

When slides were blocked with HIFCS alone, background was high (not shown). Therefore the threshold for specific binding was high, and subsequently CD20⁺ cells could not be easily detected above the background (figure 3a). When slides were blocked with Fc blocker and HIFBS, background was low and bright surface staining of CD20 could be easily detected (figure 3b). It was concluded that Fc blocker should be used for subsequent staining.

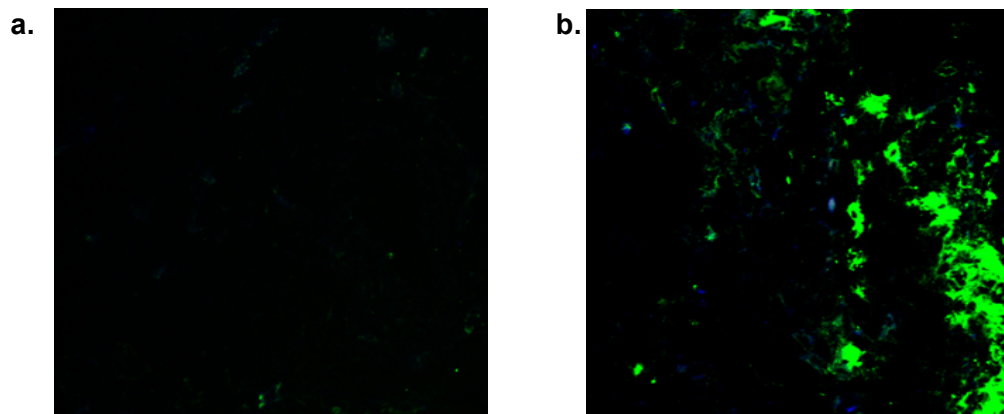


Figure 3 The effect of blocking slides with Fc blocker prior to staining synovial tissue. **(a)** Sections stained for CD20 without blocking with an Fc blocker **(b)** Section was blocked with an Fc blocker and stained for CD20.

Unlabelled anti-FcRL4 antibody should be used at 5µg/ml

To alleviate spectral bleed-through, the PECy7-conjugated anti-FcRL4 antibody was replaced with an unlabelled anti-FcRL4 antibody. In order to assess the appropriate concentration of anti-FcRL4 antibody to use for staining of synovial tissue biopsies, an antibody titration was carried out on tonsil tissue sections. Sections were stained for CD20 (green) and FcRL4 (blue). Anti-CD20 was added at 3.5µg/ml and anti-FcRL4 was tested at 10µg/ml, 5µg/ml, 1 µg/ml and 0.5µg/ml. The slides were blocked with 10%Fc blocker before staining.

In all sections bright CD20 staining was seen, and background levels were low. FcRL4 staining was bright at 10µg/ml and 5µg/ml, but could not be detected at 1µg/ml or 0.5µg/ml. Although results suggest that the unlabelled FcRL4 antibody could be used at either 10µg/ml or 5µg/ml, it was concluded that it should be applied at 5µg/ml to reduce costs (figure 4).

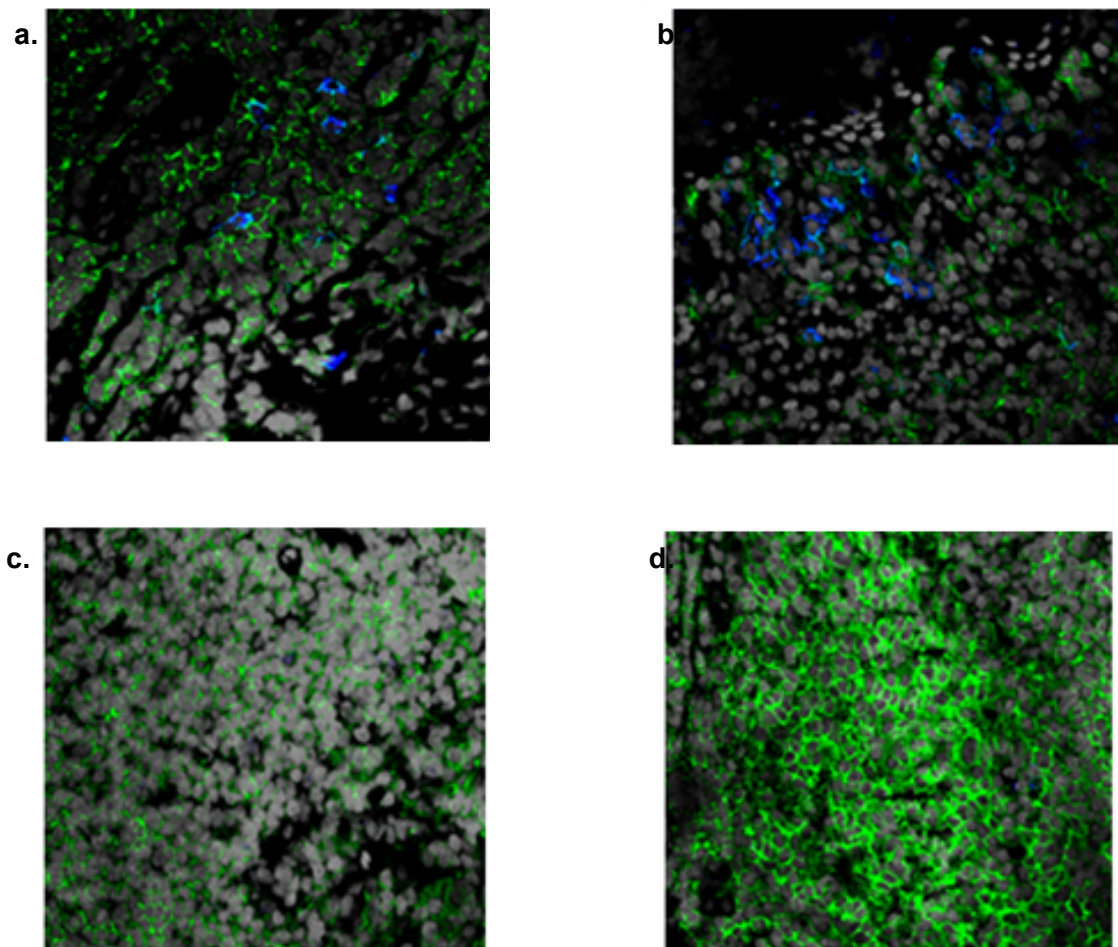


Figure 4. Unlabelled anti-FcRL4 antibody titration. Tonsil tissue sections were stained for CD20 (green) and FcRL4 (blue). Panels show CD20, FcRL4 and nuclear counterstain overlaid. **(a)** Anti FcRL4 antibody added at 10µg/ml **(b)** Anti FcRL4 antibody added at 5µg/ml **(c)** Anti FcRL4 antibody added at 1µg/ml. **(d)** Anti FcRL4 antibody added at 0.5µg/ml.

Rhodamine secondary antibody enables easy identification of FcRL4⁺ B cells.

FcRL4⁺ B cells are a relatively rare population and are therefore difficult to locate within the synovial tissue biopsies. FcRL4 staining was previously developed with Cy5 secondary antibodies which fluoresce blue. Use of this secondary antibody compromised detection of FcRL4⁺ CD20⁺ B cells because it was not possible to perfectly focus the microscope on both cell surface markers simultaneously. Furthermore, locating FcRL4⁺ cells was impeded because cells which fluoresce blue cannot be seen through the eyepiece of the confocal microscope, and scanning through the tissue section using the camera of the microscope is a much slower process. To solve these problems the Cy5 secondary antibody was replaced with a Rhodamine secondary antibody which fluoresces red. Tonsil tissue sections were stained for CD20 (green) and FcRL4 (blue/red).

Bright CD20 and FcRL4 staining was detected in tonsil with Rhodamine or Cy5 secondary antibodies. When Cy5 was used it was not possible to sharply focus on both FcRL4 and CD20 staining at the same time and therefore co-localisation was difficult to detect (figure 5a). When Rhodamine was used it was possible to focus on both FcRL4 and CD20 staining and co-localisation could be seen (yellow) (figure 5b).

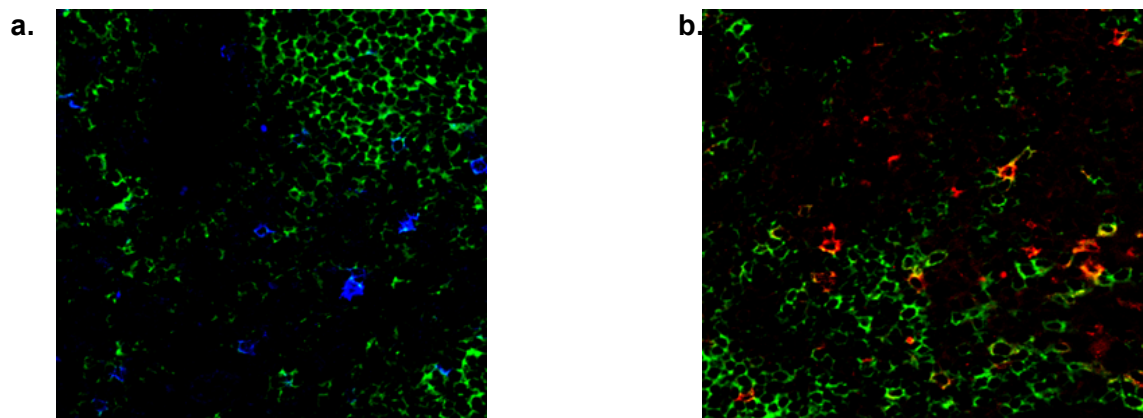


Figure 5. Detection of FcRL4⁺ B cells in tonsil tissue. Sections were stained for CD20 (green) and FcRL4. Panels show CD20 and FcRL4 overlaid. **(a)** FcRL4 staining developed with a Cy5 (blue) secondary antibody **(b)** FcRL4 staining developed with but was later developed with a Rhodamine (red) secondary antibody.

Prevalence of FcRL4⁺ B cells could not be quantified

The optimised protocol was then tested on synovial tissue samples from 10 patients with established or early RA, or resolving synovitis. Sections were stained for CD20, FcRL4 and RANKL or TNF- α . Both Cy5 and Rhodamine secondary antibodies were tested for developing FcRL4 staining. Slides were imaged using LSM 510-UV and Zen confocal microscopes. Representative confocal images from sections from patients with established RA are shown.

In all appropriate sections from patients with established RA, RANKL was brightly stained (figure 6a-c). Localisation was not limited to B cells, suggesting that RANKL was also made by other cell populations. CD20 staining varied dramatically between patients. In some sections large B cell infiltrates were seen (figure 6c-d), while in other sections only very few B cells were identified (figure 6a-b). TNF- α was not identified in sections from any patients (figure 6d). FcRL4 staining was seen in some sections; however it was not co-localised with CD20 staining (figure 6c-d). It was not possible to consistently visualise FcRL4 staining developed with either Cy5 or Rhodamine, on either the LSM 510-UV confocal or Zen microscope. FcRL4 staining was not detected on sections from patients with early arthritis (images not shown).

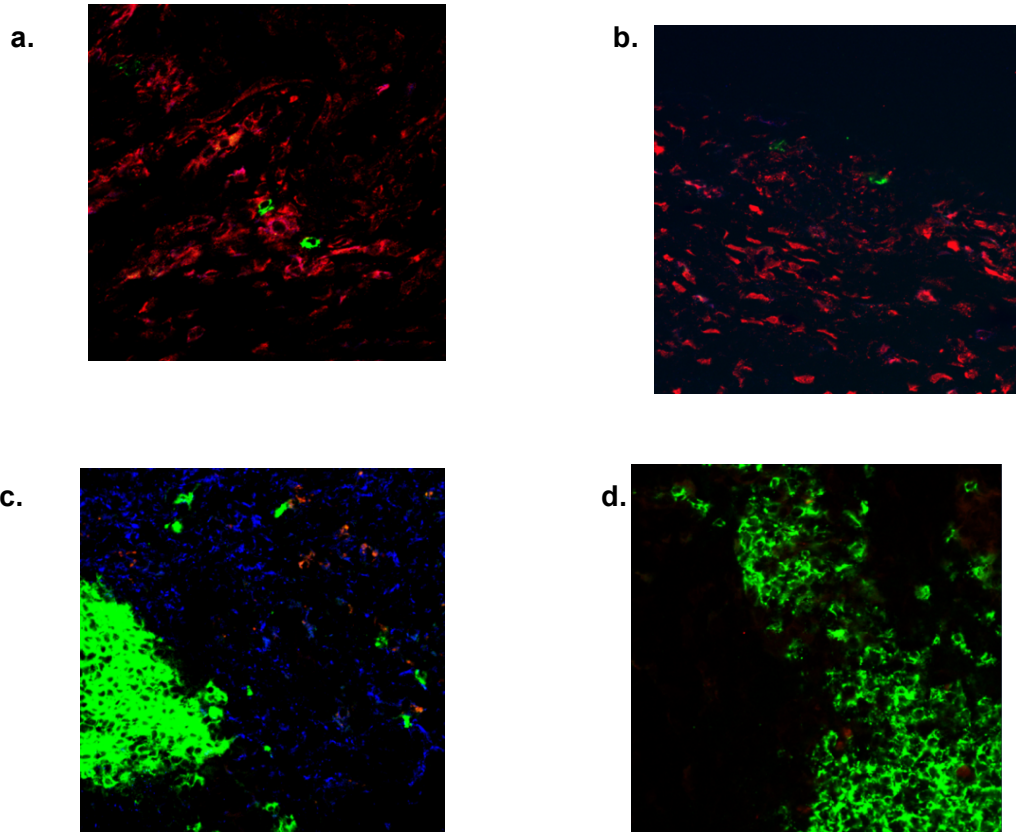


Figure 6. Detection of FcRL4⁺ B cells in synovial tissue from patients with established RA. Representative panels showing CD20, FcRL4 and RANKL or TNF-α staining overlaid. **(a)** Sections stained for CD20 (green) RANKL (red) and FcRL4 (blue) imaged using the LSM 510-UV confocal microscope **(b)** Section stained for CD20 (green) RANKL (red) and FcRL4 (blue) imaged using the Zen microscope. **(c)** Section stained for CD20 (green) RANKL (blue) and FcRL4 (red) imaged using the LSM 510-UV confocal microscope. **(d)** Section stained for CD20 (green) TNF-α (blue) and FcRL4 (red) imaged using the Zen microscope.

18S and beta-actin are suitable housekeeping genes for q-PCR analysis

Using the 7900HT PCR machine, qPCR was used to detect mRNA expression of FcRL4 from PBMC, SFMC and synovial tissue of patients with RA and other rheumatic diseases. Before qPCR experiments were carried out on patient samples, a suitable housekeeping gene was identified. The transcript levels of housekeeping genes 18S, beta-actin and GAPDH were analysed on decreasing PBMC numbers from one healthy donor. cDNA isolated from 2.5×10^5 , 1×10^5 and 6.25×10^4 PBMCs were added per reaction, and reactions were carried out in duplicate to ensure reliability of results.

All housekeeping genes were detected in all samples. For 18S and beta-actin, RQ values correlated with cell number. RQ values did not correlate with cell number for GAPDH. Therefore, 18S and beta-actin make suitable housekeeping genes for subsequent experiments. Due to availability, 18S was selected for use in subsequent experiments.

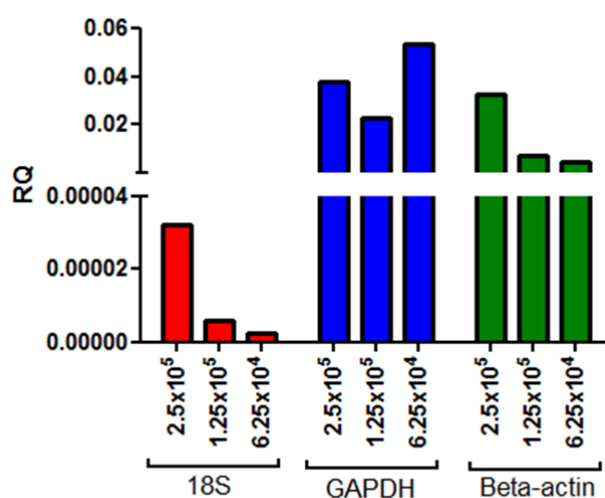


Figure 7. mRNA expression determined by qPCR on PBMCs isolated from a healthy donor to identify a suitable housekeeping gene. GAPDH, beta-actin and 18S tested on DNA extracted from decreasing cell numbers (2.5×10^5 , 1×10^5 and 6.25×10^4 cells per reaction).

DNA extracted from 62,500 cells should be used in each PCR reaction

In order to identify the optimal cDNA concentration to be used for individual qPCR reactions cDNA was extracted from an increasing number of cells and tested. cDNA from 5×10^5 , 2.5×10^5 , 1.25×10^5 , 6.25×10^4 and 3.125×10^4 cells were tested for 18S expression.

DNA sample is overloaded when 5×10^5 or 2.5×10^5 cells are added per reaction and 18S is not amplified. 18S is amplified when 1.25×10^5 and fewer cells are added per reaction. Some synovial fluid samples may contain few SFMCs and so, to ensure consistency, 6.25×10^4 was selected as an appropriate cell number to use per reaction.

Cell number/reaction	RQ
500,000	N/A
250,000	N/A
125,000	1.89×10^{-7}
62,500	5.66×10^{-8}
31,250	1.3×10^{-7}

Table 1. qPCR titration of cDNA extracted from PBMCs isolated from a healthy donors and tested for 18S. Table showing cell number from which the cDNA was extracted and mRNA expression of 18S.

FcRL4 mRNA expression is significantly elevated in synovial tissue from patients with established RA

The mRNA expression of FcRL4 in synovial tissue biopsies taken at the early arthritis clinic was investigated. Biopsies were taken from normal patients, from patients with established RA and from patients with early arthritis. Disease outcome of the patients with early arthritis was tracked, but at the time of biopsy disease was not established. Following the biopsy, patients were followed up for up to 18 months. At this point they were classified as having RA according to the 1987 ACR criteria, an alternative persistent inflammatory arthritis (eg psoriatic arthritis), an unclassified persistent arthritis or a resolving arthritis. Resolving arthritis was defined as the absence of clinically apparent synovial swelling at final assessment with no DMARDs or steroids having been used for the previous three months. Statistical analysis was carried out by Mann-Whitney test and $p < 0.05$ was considered to be significant.

No FcRL4 mRNA expression was seen in samples taken from normal and non-RA persistent patients. Significantly elevated FcRL4 mRNA expression was detected in tissue samples from synovial tissue samples from patients with established RA compared to normal donors ($p = 0.0295$). However, FcRL4 mRNA was not identified in all individuals with established RA. FcRL4 mRNA expression was seen in some individuals with early resolving arthritis and early RA.

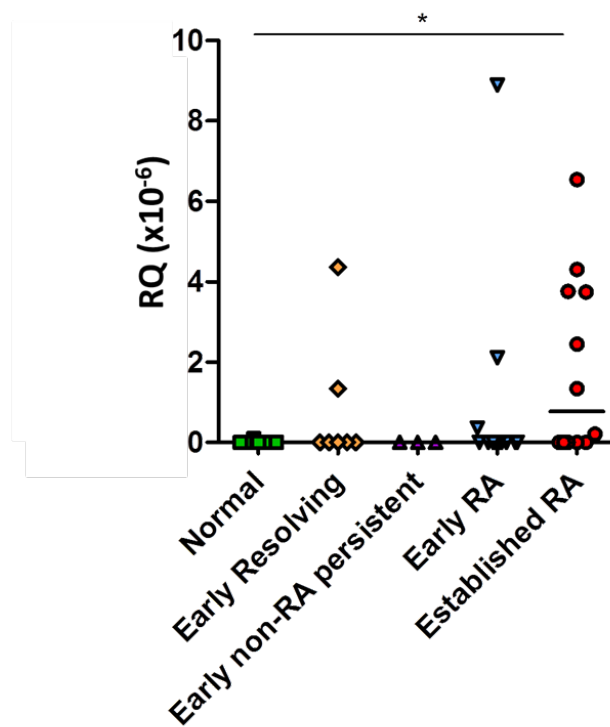


Figure 8. FcRL4 mRNA expression determined by qPCR on cDNA extracted from synovial tissue biopsies from healthy donors and arthritis patients from the early arthritis clinic. Biopsies were taken from patients with resolving arthritis, persistent arthritis that was not RA and RA prediagnosis (early) and patients with established RA. Statistical significance was assessed by Mann-Whitney test * $p < 0.05$.

FcRL4 mRNA expression is elevated in SFMCs from patients with established RA

The mRNA expression of FcRL4 in PBMCs and SFMCs was investigated. Peripheral blood and synovial fluid samples were taken from healthy donors, patients with established RA, patients with early arthritis and patients suffering from other rheumatic diseases. DNA from 6.25×10^4 cells was added per reaction.

FcRL4 mRNA was not expressed in PBMCs from healthy donors. We found FcRL4 mRNA expression above levels detected in healthy donors in 3 out of 8 PBMCs and 4 out of 8 SFMCs from patients with established RA. Patients with early arthritis showed slightly elevated transcript levels compared to healthy donors, but n numbers were very low. Patients with other rheumatic diseases did not express FcRL4 mRNA with the exception of one patient with ankylosing spondylitis.

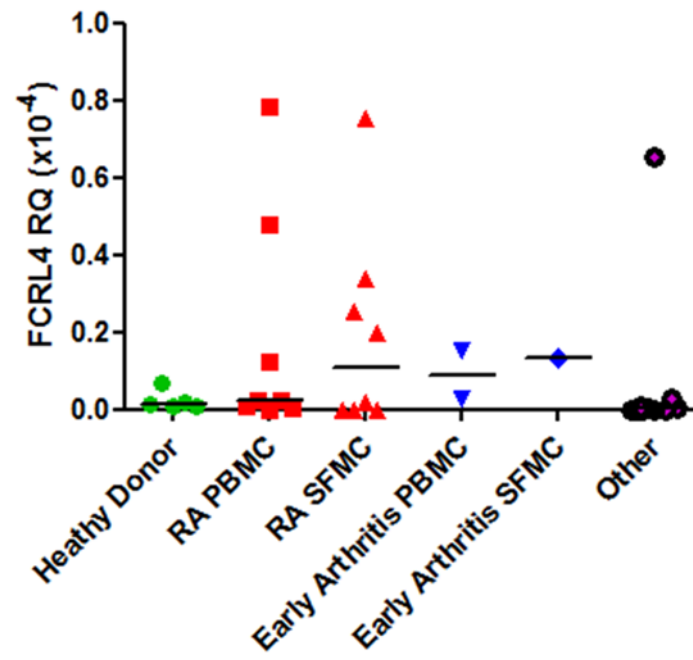


Figure 9. mRNA expression of FcRL4 determined by qPCR on cDNA extracted from PBMCs and SFMCs from healthy donors, patients with established RA and patients with early stage arthritis or other rheumatic diseases.

Discussion

This project has focused on a distinct subset of memory B cells in rheumatoid arthritis characterised by the expression of the transmembrane protein FcRL4. FcRL4⁺ B cells have been previously identified in epithelial surfaces where they have been shown to produce RANKL, but have never before been described in RA [32]. We have investigated the prevalence of this population of B cells in the synovium and peripheral blood of RA patients using a combination of confocal microscopy and qPCR, and have attempted to assess their role in disease pathogenesis by staining for RANKL and TNF- α . These results could have implications for the development of novel therapeutic strategies which are more targeted than currently used B-cell depleting therapy and do therefore not leave the patient immunocompromised. Furthermore, identification of FcRL4⁺ B cells in peripheral blood or synovial fluid could be useful for the development of a minimally invasive PCR-based test for early and accurate diagnosis of RA.

Optimisation of the protocol for confocal imaging of FcRL4⁺ B cells and TNF- α proved to be more complicated than was expected. The most significant problem encountered was high background staining on the negative control slides. The non-specific staining encountered was not localised to cellular structures but was randomly distributed throughout the tissue sections. Microscope settings for imaging tissue sections were set to ensure that the negative control slides showed no fluorescence. Subsequently specific antibody binding could not be detected above the negative threshold set, despite being clearly localised on cells when a lower threshold was used. Although an established protocol for CD20 staining was followed, specific staining was barely detectable above background levels in tissue sections. TNF- α staining of activated macrophage cytopins demonstrated that the use of high antibody concentrations may not be advantageous for the detection of specific responses as increasing concentration of the primary antibody resulted in increased non-specific binding in matched isotype negative controls. Background fluorescence was successfully reduced by blocking the slides with an Fc receptor blocker and specific staining could

consequently be detected. The Fc blocker inhibits the Fc region of monomeric IgG antibodies binding to Fc receptors which are present on immune cells such as macrophages.

Immunofluorescence microscopy highlighted the heterogeneity of the RA synovium. Notably, there were dramatic differences seen between frequencies of CD20⁺ B cell numbers between patients, all of whom had established RA. Although the presence of B cells may not alone drive disease, the prevalence of B cells in the synovial tissue may correlate to rituximab treatment efficacy, and could be useful for the selection of candidates for this treatment. Improving the selection criteria for rituximab treatment would mean that patients would not be unnecessarily depleted of B cells.

RANKL, which causes osteoclast formation, is known to be vital for driving bone destruction in RA. In accordance with previous studies, staining of RANKL was identified in, but was not restricted to, B cells in the synovium [29]. Staining for this potent cytokine highlighted its broad localisation throughout the tissue of patients with established RA. It is possible that B cells may be a key source of the RANKL seen dispersed throughout the tissue. Interestingly, treatment with rituximab has been shown to alleviate joint destruction, which suggests that these CD20⁺ cells expressing RANKL may be significant in disease pathogenesis[31].

TNF- α is a fundamental inflammatory cytokine involved in disease progression of RA. In order to investigate the ability of B cells in the synovial tissue to produce TNF- α , staining was first optimised using cytopins of activated macrophages. Surprisingly, TNF- α was limited to relatively few macrophages on the cytopins, suggesting that not all of the macrophages had been successfully stimulated. Alternatively, the cytopins may have been prepared before the cells started producing the cytokine, or after TNF- α had diffused out of the cell into the surrounding medium and was therefore too dispersed to get significant staining. No surface or extracellular staining was seen

and TNF- α was contained within the cell which suggests is localised in the Golgi body [40]. It was not possible to detect staining in any synovial tissue biopsies from patients with established RA. This result was surprising as TNF- α is known to significantly contribute to RA pathogenesis by exacerbating inflammation and inducing bone destruction in the joint[17, 22]. It is possible that TNF- α was present but specific staining was weak and could not be detected above the level of the isotype negative control.

It was not possible to use immunofluorescence microscopy to identify FcRL4⁺ B cells in sections of synovial tissue biopsies taken from patients with established or early arthritis. This could suggest that FcRL4⁺ B cells are not localised in the RA synovium and are therefore not significant in disease pathogenesis. However, qPCR data from this project has shown increased FcRL4 mRNA expression in cells from synovial tissue from patients with established RA compared to healthy controls. Therefore, further work should focus on improvement of the staining protocol for FcRL4. Single staining of tissue biopsies for FcRL4 without CD20, TNF α or RANKL staining may reduce background and enable a lower threshold to be set for specific staining. Amplification of staining could be achieved using biotin-streptavidin complexes[41]. Alternatively, Alexa Fluor labelled secondary antibodies could be tested as the staining should be brighter and easier to detect above the negative isotype control. Once identified, FcRL4 staining should be quantified so that prevalence of these cells can be compared between patients, and disease types. This can be achieved using the Zen confocal microscope which can perform tile scans, and will therefore be able to identify all FcRL4⁺ cells throughout the entire tissue section.

qPCR analysis of synovial tissue biopsies showed significantly increased FcRL4 mRNA expression in cells from patients with established RA compared to healthy controls, suggesting a role for FcRL4⁺ B cells in disease progression. Expression was not elevated in all tissue samples from established RA patients, reflecting the heterogeneity of RA. FcRL4 mRNA expression detected by

qPCR did not correspond to FcRL4 staining which may be attributed to errors in the staining protocol. Alternatively translation of FcRL4 mRNA may be intrinsically regulated. FcRL4 mRNA expression was also elevated in some patients with early RA but not patients with early non-RA persistent arthritis which suggests that FcRL4⁺ B cell population in the synovial tissue may be restricted to RA patients. More patient samples must be collected and tested before firm conclusions can be drawn. Differences may be accounted for by variation in cellular compositions between samples. Some samples may contain relatively few immune cells compared to fibroblasts and exact location from which the biopsy was taken was not standardised. To compensate for this, sensitivity of the qPCR protocol should be optimised to ensure complete and accurate detection of FcRL4 mRNA. This could be improved by designing and testing new primers for FcRL4 in preference to primers bought from Applied Biosystems.

Investigation of mononuclear cells from synovial fluid and peripheral blood of patients with established RA show elevated expression of FcRL4 mRNA in some SFMCs and PBMCs compared to healthy donors, again results reflected the divergence of the disease. Although the increases seen were not significant, further samples should be collected and tested so that firm conclusions may be drawn. FcRL4 mRNA expression was increased in a patient with ankylosing spondylitis which corresponds with reports of FcRL4 mutations conferring disease susceptibility and again raises the question of whether FcRL4⁺ B cells may also be implicated in progression of other chronic inflammatory diseases[39]. More PBMC and SFMC samples should be tested from patients with early arthritis to determine the significance of FcRL4⁺ expression and further work should include longitudinal studies which track the expression of FcRL4 from early arthritis patients as they progress into disease.

Functional and *in vivo* assays could be used to elucidate the role of FcRL4⁺ B cells in RA. Although mice do not express FcRL4, an *in vivo* mouse/human system could be developed to test the

capability of anti-FcRL4 antibodies to activate or deactivate FcRL4⁺ B cells and the effect of this on RA severity. This could be significant for the development of new RA treatments which target only this population of B cells, thus alleviating severe complications associated with current treatment.

Confirmation that FcRL4 mRNA expression is found in patients with early arthritis and is limited to patients who go on to develop established RA, could prove useful for the early diagnosis of RA. Preliminary qPCR results described in this investigation suggest that FcRL4 may be up regulated in the synovial fluid and peripheral blood of RA patients, highlighting the potential for the development of a novel PCR based diagnostic test for RA. In conjunction with current diagnostic methods this could be used for accurate diagnosis of RA before the onset of severe symptoms. Current B cell depletion therapy, rituximab, targets all CD20⁺ cells and renders the patient severely immune deficient[30] Using qPCR we have shown elevated presence of FcRL4⁺ B cells in the synovial tissue of patients with established RA compared to healthy controls. This allows speculation that FcRL4 may be significant in disease pathogenesis and could be a potential target for improved treatment. Combined, the results of this project suggest a role FcRL4⁺ B cells in the development of RA, the nature of which must be further investigated but may contribute to improved diagnosis and treatment of this debilitating disease.

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